Carbon Disulfide Neurotoxicity Defined

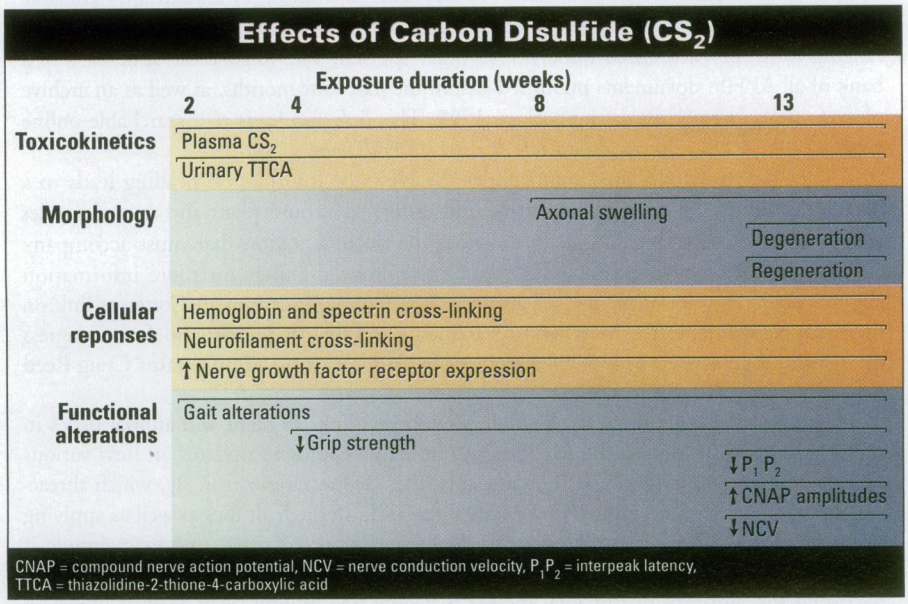
In 1997, a team of researchers based at the NIEHS was awarded a Bronze Medal for Commendable Service by the EPA for a series of studies on the neurotoxicity of carbon disulfide (CS_2). Performed in collaboration with scientists from the EPA, Duke University Medical Center in Durham, North Carolina, and the University of North Carolina at Chapel Hill, the studies are unique in their comprehensive examination of the sensitive biochemical, functional, and structural changes to the nervous system resulting from exposure to CS_2. The findings have provided valuable insight into the processes underlying CS_2 neurotoxicity, and offer a model for the study of effects of exposure to other toxic compounds.

CS_2 has been used since the early nineteenth century as a solvent in the manufacture of sulfur matches and in the extraction of fats. It has also been used in the cold vulcanization of rubber; clinical signs of nerve damage associated with both acute and chronic exposures were first described in rubber industry workers. Cold vulcanization was eventually replaced by other processes that do not use CS_2, but the compound is still used today in the production of rayon and cellophane from wood fiber (because CS_2 serves as a reactant in this process, replacement with a nontoxic chemical has not been possible). The U.S. Department of Health and Human Services estimates total atmospheric discharges of CS_2 to be approximately 76 million pounds per year.

While rayon plant workers are at direct risk of exposure to CS_2, other populations may be exposed through indirect means. Dithiocarbamates and their disulfides, chemicals that are widely used in pesticides and as therapeutic agents for conditions including cancer and drug addiction, can liberate CS_2 upon decomposition within the body. Thus, people who apply pesticides or harvest produce sprayed with pesticides, as well as persons consuming therapeutics such as disulfiram (Antabuse), may also be exposed to CS_2.

The major route of human exposure to CS_2 is inhalation. Absorbed CS_2 is taken up by the blood and distributed throughout the body. Acute exposure to high concentrations of CS_2 may result in euphoria, hallucinations, irritability, manic delirium, and convulsions. Prolonged exposure to low concentrations of CS_2 in air can damage both the structural and functional integrity of nerves, particularly affecting long, large-diameter, myelinated axons in both the central and peripheral nervous system. More than a dozen studies have characterized the morphologic changes in the nervous system resulting from different levels of exposure. However, prior to the NIEHS studies, the various stages of neurotoxicity had yet to be examined in a coordinated manner.

As part of the Clean Air Act Amendments of 1990, the EPA listed CS_2 as a high priority agent for further evaluation. The EPA issues risk assessment guidelines for chemicals such as CS_2, and the agency continuously seeks to refine such guidelines as the science for understanding that risk progresses. The agency requested that the NIEHS undertake studies that would aid in setting more appropriate mechanistically based exposure standards.



The endpoints. Researchers summarized the CS_2-induced alterations in F344 rats exposed to 800 ppm CS_2.
Source: Harry GJ, Graham DG, Valentine WM, Morgan DL, Sills RC. Carbon disulfide toxicity in rats: VIII. Summary. Neurotoxicology 19(1):159–162 (1998).

Study Design

Robert Sills, head of molecular pathology in the NIEHS Environmental Toxicology Program, was given the job of coordinating the CS_2 studies along with Daniel Morgan, head of the NIEHS inhalation laboratory, and Jean Harry, head of the institute's neurotoxicology group. The researchers began in 1993 by identifying the data gaps in the knowledge about CS_2 toxicity, then sought out the expertise needed to conduct the additional research. As it happened, that expertise lay close at hand. Also at the NIEHS were Michael Moorman and Bradley Collins, experts on toxicokinetics. Doyle Graham and William Valentine, then a professor and an assistant professor of pathology, respectively, at the Duke University Medical Center, had both previously authored studies on the mechanisms of CS_2 toxicity. Arrel D. Toews, a research professor of biochemistry at the University of North Carolina at Chapel Hill, had also conducted research in CS_2 neurotoxicity. Finally, toxicologists from the EPA's neurotoxicology division in Research Triangle Park, North Carolina, including Virginia Moser, a specialist in neurobehavioral studies, and David Herr, an expert in neurophysiology techniques, were also available to work on the studies. The proximity of the researchers' four institutions enabled the full range of studies to be conducted at the NIEHS inhalation laboratory using one group of animals. This resulted in tremendous cost savings and increased the confidence in the results.

"It was very critical to the success of the project to be able to conduct all the studies at one place using the same animals," Sills says. "That way, we knew the animals were exposed to the exact same dosages and kept under the same experimental conditions. The same scientific staff and equipment were employed throughout the study, all of which cuts way down on the variables. Inhalation studies are very expensive, costing $150,000–200,000 by some estimates. Because we were able to use the same animals for each of the six studies, we were able to save a tremendous amount of money."

The studies were designed to assess multiple biological and mechanistic endpoints at various time periods of subchronic exposure. Researchers used Fischer 344 rats, a strain used in previous CS_2 inhalation studies. Rats were exposed to either 0, 50, 500, or 800 parts per million (ppm) CS_2 for 6

hours per day, 5 days per week, for 2, 4, 8, or 13 weeks.

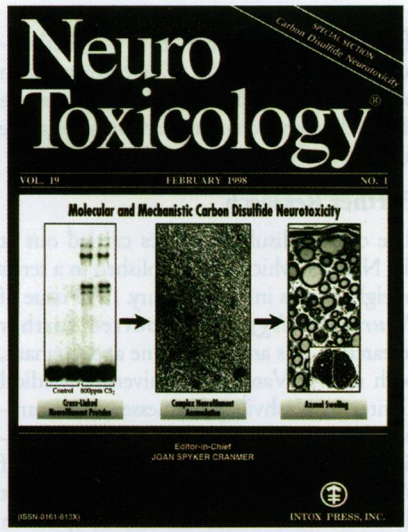
An important consideration in designing the studies was the need to correlate the inhaled CS_2 dose and the neurobehavioral, neurophysiological, and neuropathological effects. To do this, it was essential that all the endpoints be obtained on all of the animals. These endpoints included pharmacokinetic changes in blood CS_2 levels and urinary metabolites, blood and spinal cord biomarkers of exposure and effect, neurotoxicity alterations in axon–Schwann cell interactions, pathology of the peripheral nerve and spinal cord, nerve conduction velocity (NCV), compound nerve action potential (CNAP), and behavioral assessments using a Functional Observational Battery (FOB). Immediately following exposure on the day prior to the penultimate exposure, urine was collected for metabolic analysis. During and after the next inhalation exposure, blood was collected for analysis of CS_2 and metabolites. Following the last exposure, rats were examined by FOB and then assessed for electrophysiological function. Next, 5 rats per sex per dose group were evaluated for the presence of CS_2 protein–protein cross-linking in the red blood cells and the neurofilaments of the spinal cord. Segments of the sciatic nerve were collected and analyzed for alterations in the low-affinity nerve growth factor receptor (NGF-R) mRNA. Four rats per sex per dose group were selected for the morphologic evaluations.

Results

One of the prerequisites for characterizing the toxicity of any compound is to understand how the compound is taken up by the body and either retained or eliminated. The concentration and duration of exposure and the number of previous days of exposure to the compound all can affect the amount that is actually retained. To evaluate the relationship between measures of the inhalation exposure to CS_2 and the toxicologic response and biomarkers of exposure, the research team performed three separate studies characterizing CS_2 kinetics in the test animals.

A single exposure inhalation study was conducted to investigate the uptake and elimination kinetics of CS_2. A single-exposure intravenous study was also conducted to estimate the volume of distribution and total systemic clearance. Finally, a 13-week inhalation study involving repeated exposures characterized the plateau of CS_2 blood levels and 2-thiothiazolidine-4-carboxylic acid (TTCA) excretion.

The studies found that, at the concentrations tested (50, 500, and 800 ppm), there is not a linear dose–response relationship between the amount of CS_2 inhaled and either blood CS_2 concentration or urinary TTCA excretion. Both blood CS_2 and urinary TTCA became saturated at these higher levels. Thus, these measures appear to be useful only as indicators of exposure to relatively low levels of CS_2 exposure and short exposure time frames.



The results are in. The award-winning CS_2 studies were published in a special section in the February 1998 issue of *Neurotoxicology*.

Using samples obtained from the NIEHS collaboration, it was demonstrated that covalent cross-linking of neurofilament proteins was a direct effect of CS_2 contributing to the formation of neurofilamentous axonal swellings, a lesion characteristic of CS_2 neurotoxicity. It was also shown that cross-linking of neurofilament proteins was positively correlated to CS_2-mediated covalent cross-linking of spectrin, a red blood cell membrane protein. A linear dose–response relationship was observed for these two biochemical events, suggesting that populations exposed to CS_2 could be evaluated through periodic blood sampling, and that the quantity of spectrin cross-linking could be used to identify people in danger of developing neurotoxicity. Hemoglobin has a similar biological life to that of erythrocyte spectrin, and is easier to isolate and analyze. As part of the NIEHS research, scientists sought to evaluate modified hemoglobin as a potential dosimeter for quantifying exposure to CS_2.

The study found that hemoglobin modification does, indeed, possess several advantages over spectrin cross-linking as a biomarker of effect for CS_2 exposure. Modification of hemoglobin can be detected at earlier time points than spectrin dimer formation. Analysis of hemoglobin requires drawing far less blood than that of spectrin and can be performed with more direct and rapid methods. Collectively, the findings suggest that alterations in the alpha chain of hemoglobin may provide a sensitive neurotoxic biomarker of effect for CS_2 with the potential to provide mechanistically relevant assessments of subchronic exposures, a tool to help identify susceptible individuals, and a means to examine possible effects occurring at presently acceptable levels of CS_2 exposure.

Researchers also examined the potential of using mRNA expression of NGF-R as an early indicator of peripheral nervous system damage. One of the effects of chronic CS_2 exposure is the retraction of myelin, the fatty substance that ensheathes nerve fibers. Previous research had shown that mRNA levels are markedly upregulated in the sciatic nerve during demyelination. Upregulation also occurs in various subdegenerative axonopathy models where there is axonal atrophy. These findings suggested that mRNA upregulation could be a useful biomarker for subtle perturbations in normal axon–Schwann cell interactions. To further test this hypothesis, the team examined NGF-R mRNA expression in sciatic nerves of rats exposed to CS_2.

The study revealed that NGF-R mRNA expression does increase in a dose- and time-dependent manner. Morphologic alterations in the sciatic nerve were not apparent, even at the highest dosages with the longest exposure times. Thus, upregulation of NGF-R mRNA is an indicator of subtle alterations in the normal axon–Schwann cell relationship and does provide a sensitive measure of CS_2 neurotoxicity. Researchers state that the assay of this marker may also be useful as a rapid and sensitive general screen for other compounds that are potentially toxic to the peripheral nervous system.

One of the most widely reported findings in people and animals exposed to CS_2 is neurotoxicity in the central and peripheral nervous system. Yet few studies have fully examined the morphologic progression, biology, and mechanism of CS_2-induced neurotoxicity. Therefore, the researchers conducted a study to examine the progression and dose response of CS_2 distal axonopathy by light and electron microscopy and in teased nerve fiber preparations. They then correlated these observations with other biologic and mechanistic findings using inhalation studies.

The study revealed that both behavioral changes and biochemical effects, such as cross-linking of hemoglobin and neurofilament proteins, and increases in NGF-R mRNA expression occur prior to axonal swelling. The study illustrates that the detection of neurotoxic effects prior to

morphologic changes can be used to discern potential neurotoxicity and mechanisms of toxicity.

A frequently cited functional change following CS_2 exposure is a decrease in NCV. The researchers hypothesized that alterations in peripheral nerve function produced by CS_2 exposure would be reflected in changes in CNAP and/or NCV. Using electrophysiological testing and microscopic examination of the ventral caudal tail nerve, researchers quantified concentration- and time-related changes in peripheral nerve electrophysiology produced by subchronic exposure to CS_2. This study revealed that exposures to 500 ppm or 800 ppm CS_2 for 8–13 weeks produced some minor changes in NCV and CNAP recorded from the ventral caudal tail nerves of experimental animals. The biological basis for the changes in CNAP produced by CS_2 is currently under investigation.

A final study measured behavioral changes in test animals using a FOB. Neuromuscular deficits, including gait alterations and decreased hindlimb and forelimb grip strength, were detected in the test animals after as little as two weeks' exposure to 800 ppm CS_2. These changes were closely related to CS_2 concentration and exposure duration. Other effects, observed mostly at 13 weeks, included decreased responsiveness to a visual stimulus and mild tremors. Correlations with other endpoints in the project demonstrate that behavioral changes can be a sensitive indicator of CS_2 neurotoxicity.

Further Research

The carbon disulfide studies carried out at the NIEHS, which were published in a series of eight papers in the February 1998 issue of *Neurotoxicology*, have spurred further research in this area. Valentine and Graham, both now at Vanderbilt University Medical Center in Nashville, Tennessee, used samples from the NIEHS studies in a study published in the January 1997 issue of *Toxicology and Applied Pharmacology* that demonstrated the sensitivity of spectrin cross-linking for detecting inhalation exposures to CS_2, and provided evidence for covalent cross-linking of neurofilament proteins as a mechanism for CS_2-induced axonal neurofilamentous swellings. They also used the biomarkers developed in the CS_2 inhalation study in a second study, published in the February 1998 issue of the same journal, to quantify the amount of CS_2 liberated by diethyldithiocarbamate *in vivo*. A third study, published in the May 1998 issue of *Chemical Research in Toxicology*, established the formation of CS_2-mediated thiourea protein cross-links on spectrin *in vivo*.

Valentine and Graham now intend to investigate the utility of CS_2-mediated protein modifications for monitoring high-risk human populations. "The information derived from the human studies should indicate how useful our biomarkers are for developing intervention strategies for identifying and removing individuals from neurotoxic levels of CS_2 or compounds that release CS_2 prior to their developing neurological deficits," says Valentine.

The EPA, meanwhile, anticipates using the data from the CS_2 studies for a series of actions defined under the Clean Air Act Amendments of 1990. "These studies will be vital to the EPA for development and refinement of mechanistically based quantitative human health risk assessments for chemicals listed in the Clean Air Act," says Gary Foureman, a health scientist with the EPA's National Center for Environmental Assessment in Research Triangle Park. "These risk assessments, in turn, are used for such measures as the listing and delisting of hazardous air pollutants, setting of emission standards through Maximum Achievable Control Technology, and providing technical assistance through air toxics clearinghouses such as the Air Risk Information Support Center."

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